# Detection of Circulating Tumor Cells in Colorectal and Gastric Cancer Using a Multiplex PCR Assay

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**Abstract.** *Aim: The aim of this study was the development of a multiplex-PCR assay for the detection of circulating tumor cells in patients with colorectal and gastric cancer. Patients and Methods: Peripheral blood samples were collected from 81 patients with colorectal cancer, 16 with gastric cancer and 38 healthy blood donors, as controls. The samples were processed for RNA extraction and cDNA synthesis and were subsequently analyzed for the expression of cytokeratin 19 (CK19), cytokeratin 20(CK20) and epidermal growth factor receptor (EGFR) with multiplex PCR. Results: Statistical analysis revealed that the combination of CK19 and CK20 could be useful in the exclusion of colorectal cancer, as well as the diagnosis and exclusion of gastric cancer. Furthermore, the expression of EGFR was correlated with the presence of systemic disease in patients with colorectal cancer. Conclusion: Multiplex-PCR-based detection of circulating tumor cells could serve as a useful tool for the diagnosis, and monitoring of patients with colorectal and gastric cancer.*

Colorectal cancer (CRC) is a multi-step disorder that involves the accumulation of genetic as well as epigenetic aberrations. The disease initiates in most cases from a benign adenomatous polyp, which accumulates mutations in a wide range of genes, progressing into a dysplastic polyp and finally into cancer with metastatic potential (1). CRC is a leading cause of morbidity and mortality worldwide, as it represents the third most common type of cancer in males and females, with more than 1,200,000 new cases and 600,000 deaths annually (2). Gastric cancer (GC) also represents a serious burden in global health and prosperity, with more than 900,000 new cases and 700,000 deaths annually. In general, GC is twice as common in males, at 70% of cases occurring, however, in developing countries (2). The progression of gastric carcinogenesis involves the aggregation of genetic and epigenetic abnormalities, such as telomerase activation, genetic instability, and alterations in oncogenes, tumor suppressors and cancer-related factors under the influence of environmental and lifestyle causes, including *Helicobacter pylori* and dietary components (3).

Metastasis represents the most common cause of death due to cancer-related issues, highlighting the importance of early and accurate diagnosis, prognosis and therapeutic management (4-6). The metastatic process involves generally a set of distinct steps, namely: i) epithelial–mesynchymal transition (EMT) and local invasion, ii) intravasation, iii) survival in peripheral blood and dissemination, iv) extravasation at target tissue and v) formation of distant metastases (4, 7). EMT is a dynamic and reversible process that involves the partial loss of epithelial traits from cancer cells and the acquisition of mesenchymal attributes. As a result, cancer cells gain an invasive and aggressive phenotype that drives metastasis (4, 5, 8). Although surgical resection remains the main therapeutic solution in cases of CRC and GC, a large number of patients may be diagnosed with an unresectable tumor or develop metastases after surgical interventions (5, 6, 9). Circulating tumor cells (CTCs) represent a link between the primary tumor and metastatic lesions and seem to be the main suspects responsible for the development of systemic disease and

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recurrence (5-7). Current methods for detection of CTCs include polymerase chain reaction (PCR)-based methods (DNA or RNA extraction) and cytometric methods, such as fluorescence *in situ* hybridization (FISH), fluorescence-activated flow cytometry (FACS) and the CellSearch™ test. mRNA PCR-based methods offer a relatively rapid, cost-effective and accurate isolation of CTCs (5, 9).

Candidate markers are selected according to two basic parameters: they must be as cancer-specific as possible and they must have no or only low expression in normal human leucocytes. Markers were selected according to additional criteria discussed in a previous study of our laboratory (10). After an extensive search in literature and public databases, we selected cytokeratin (CK) 19, CK20 and epidermal growth factor receptor (EGFR) as candidate markers for our study. CK19 and CK20 are members of the intermediate filament protein family and are expressed predominantly in cells of epithelial origin, and are responsible for the structural integrity of cells (5). They are expressed in a variety of cancer types, including cancer of the gastrointestinal tract (5, 6, 9). Issues regarding the specificity of cytokeratins for CTC detection that lead to false-positive results, such as expression from hematopoietic cells, amplification of pseudogenes or from the introduction of epidermal cells during blood sampling, are resolved when combining cytokeratins with other molecular markers and with careful primer design and fine-tuning of PCR conditions (5, 10). EGFR is involved in normal and malignant cell proliferation and is also expressed in a variety of cancer types of both epithelial and mesenchymal origin, including cancer of the gastrointestinal tract. EGFR is regarded as a rather specific marker of CTCs, with rare expression in hematopoietic cells, which is mainly correlated with advanced clinical stages (5, 11, 12).

The aim of the present study was the development of a multiplex-PCR assay for the detection of CTCs in patients with CRC or GC and the evaluation of its possible clinical applications.

## Patients and Methods

*Participants*. Eighty one patients with CRC [age: mean (min–max) =70 (41-92) years; 48 men and 33 women] and 16 patients with GC [age: mean (min–max)=67 (37-83) years; 8 men and 8 women) prior to surgical or any other therapeutic intervention were enrolled in the study. The patients were referred for surgical treatment. They had already been diagnosed with colorectal or gastric adenocarcinoma through gastrointestinal endoscopy and histological examination of the discovered lesions, and they had not received any treatment before. Patients were excluded from the study if they had another histological type of cancer, had received neoadjuvant chemotherapy, and had a medical history of the same or another type of cancer. The clinicopathological features of patients with CRC and GC are listed in Tables I and II, respectively. Peripheral blood samples were also collected from 38 healthy volunteers (30 men and 8 women). Six milliliters of blood was drawn from each subject, using a venous catheter, into 3 ml EDTA-containing vacutainers. The first 3 ml of each sample were discarded to avoid possible contamination with epidermal cells (10). All the participants provided written informed consent to this research protocol and this study was conducted under the approval of the local Ethics Committee and conforms to the Declaration of Helsinki.

*Primer design*. Primers for the selected markers were designed using FastPCR software (http://primerdigital.com/fastpcr.html). The sequences of the three primer pairs were additionally tested on NCBI Nucleotide Blast (http://blast.ncbi.nlm.nih.gov/blast) to confirm that they are specific for each target. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used to evaluate the performance of cDNA synthesis (housekeeping gene). The sequences of the assay primers are presented in Table III.

*Cell lines*. HT-29 colorectal adenocarcinoma cells (American Type Culture Collection number: HTB-38, Manassas, VA, USA) were used for the spiking experiments, due to the fact that they express all three markers we used in this study (13-15). HT-29 cells were maintained in Dulbecco's modified Eagle's medium/F-12 (Cambrex, Walkerville, MD, USA), supplemented with 10% heat-inactivated fetal bovine serum (Biochrom, Berlin, Germany) and 100 U/ml penicillin/streptomycin (Cambrex), at 37˚C in a humidified atmosphere of 5% $CO_2$.

*RNA isolation*. Blood samples were processed within 6 hours of collection. erythrocyte lysis buffer (ELB) was used to lyse by osmosis all erythrocyte cells and enrich the samples in nucleated leucocytes. A volume of 7.5 ml ELB (155 mM $NH_4Cl$, 10 mM $KHCO_3$ and 0.1 mM EDTA, pH 7.4) were added to each sample. The samples were kept on ice for 45 minutes, with occasional mixing by inversion and they were then centrifuged at 400× *g* for 10 min at 4˚C. The supernatant was discarded and in case red blood cells still remained in the sample, 5 ml of ELB were added to the pellet, the cells were re-suspended, kept on ice for additional 10 min and the centrifugation step was repeated. The pellet of cells was then homogenized in 1 ml Tri-Reagent RT-111 (MRC Inc., Cincinnati, OH, USA). Total cellular RNA was then extracted according to the manufacturer's instructions. Diethylpyrocarbonate (DEPC)-treated water was used for RNA pellet dilution. Total RNA concentration and quality were determined after ultraviolet spectophotometry (measurements at 260 nm, 280 nm).

*cDNA synthesis*. cDNA was synthesized using Moloney Murine Leukemia Virus (M-MLV) Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). A mixture containing 2 μg total RNA, 500 μg oligodT18 primer (Fermentas, St. Leon-Rot, Germany), 0.5 mM deoxynucleotides (HT Biotechnology Ltd., Cambridge, UK) and DEPC-treated water up to a volume of 12 μl, was heated at 65˚C for 5 min and then chilled on ice for an additional 5 min. A second mixture containing reverse transcriptase buffer (50 mM Tris-HCl/pH 8.3, 75 mM KCl, 3 mM $MgCl_2$), 10 mM dithiothreitol, 20 units Rnase OUT Recombinant Ribonuclease Inhibitor (Invitrogen), was added to the first mixture and then incubated at 37˚C for 2 min. In the next step, 150 units of M-MLV Reverse Transcriptase was added to a final volume of 20 μl, and the incubation continued at 37˚C for 50 min and the reaction was completed by an inactivation step at 70˚C for 15 min. The complementary DNA produced was then ready for multiplex PCR reaction.

Table I. *Clinicopathological features of patients with colorectal cancer.*

| Parameter | Number of cases (%) |
|---|---|
| Total cases | 81 |
| Gender (n=81) | |
| Men | 48 (59.3%) |
| Women | 33 (40.7%) |
| Age (based on median value) (n=81) | |
| <70 years | 38 (46.9%) |
| ≥70 years | 43 (53.1%) |
| Segment of the large intestine (n=81) | |
| Right | 23 (28.4%) |
| Left | 31 (38.3%) |
| Rectum | 27 (33.3%) |
| Max. tumor size | |
| (based on median value) (n=77) | |
| <4 cm | 39 (50.6%) |
| ≥4 cm | 38 (49.4%) |
| TNM stage (n=80) | |
| 0 | 5 (6.2%) |
| 1 | 23 (28.8%) |
| 2 | 24 (30%) |
| 3 | 20 (25%) |
| 4 | 8 (10%) |
| T (Tumor) (n=78) | |
| T0 | 5 (6.4%) |
| T1 | 4 (5.1%) |
| T2 | 23 (29.5%) |
| T3 | 40 (51.3%) |
| T4 | 6 (7.7%) |
| N (Node) (n=78) | |
| N0 (0 infiltrated lymph nodes) | 54 (69.2%) |
| N1 (1-3 infiltrated lymph nodes) | 15 (19.2%) |
| N2 (≥4 infiltrated lymph nodes) | 9 (11.6%) |
| M (Metastasis) (n=81) | |
| Yes | 8 (9.9%) |
| No | 73 (90.1%) |
| Grade (n=79) | |
| Low grade (1/1.5/2) | 58 (73.4%) |
| High grade (2.5/3) | 21 (26.6%) |
| Lymphovascular invasion (n=79) | |
| Yes | 15 (19%) |
| No | 64 (81%) |
| Mucinus adenocarcinoma (n=79) | |
| Yes | 8 (10.1%) |
| No | 71 (89.9%) |

Table II. *Clinicopathological features of patients with gastric cancer.*

| Parameter | Number of cases (%) |
|---|---|
| Total cases | 16 |
| Gender (n=16) | |
| Men | 8 (50%) |
| Women | 8 (50%) |
| Age (based on median value) (n=16) | |
| <67 years | 8 (50%) |
| ≥67 years | 8 (50%) |
| Segment of the stomach (n=11) | |
| Cardia | 2 (18.2%) |
| Body | 4 (36.4%) |
| Pylorus | 5 (45.4%) |
| Max Tumor size | |
| (based on median value) (n=11) | |
| <4.3 cm | 4 (36.4%) |
| ≥4.3 cm | 7 (63.6%) |
| TNM stage (n=15) | |
| 1 | 3 (20%) |
| 2 | 2 (13.4%) |
| 3 | 5 (33.3%) |
| 4 | 5 (33.3%) |
| T (Tumor) (n=12) | |
| T1 | 3 (25%) |
| T2 | 0 (0%) |
| T3 | 5 (41.7%) |
| T4 | 4 (33.3%) |
| N (Node) (n=12) | |
| N0 (0 infiltrated lymph nodes) | 4 (33.3%) |
| N1 (1-2 infiltrated lymph nodes) | 3 (25%) |
| N2(3-6 infiltrated lymph nodes) | 1 (8.4%) |
| N3 (≥7 infiltrated lymph nodes) | 4 (33.3%) |
| M (Metastasis) (n=16) | |
| Yes | 5 (31.2%) |
| No | 11 (68.8%) |
| Grade (n=14) | |
| Low grade (1/1.5/2) | 4 (28.6%) |
| High grade (2.5/3) | 10 (71.4%) |
| Lymphovascular invasion (n=11) | |
| Yes | 5 (45.4%) |
| No | 6 (54.6%) |
| Lauren's classification (n=10) | |
| Intestinal | 4 (40%) |
| Diffuse | 6 (60%) |

*PCR conditions and sensitivity of the test*. PCR reaction was performed using the Qiagen Multiplex PCR Kit (Qiagen, Hilden, Germany) in a final reaction volume of 25 μl. The reaction mixture contained 1×Qiagen Multiplex PCR Mix (HotStarTaq DNA pol, Multiplex PCR Buffer with 6 mM $MgCl_2$/pH 8.7, dNTP mix), 0.125 μM *CK19* primers, 0.125 μM CK20 primers, 0.4 μM *EGFR* primers and DEPC-treated water up to 23 μl. Complementary DNA (2 μl) was added to the reaction volume. Cycling conditions were: 95˚C for 15 min (1 cycle), 94˚C for 30 s, 57˚C for 90 s, 72˚C for 60 s (36 cycles), 72˚C for 10 min (1 cycle).

For *GAPDH* detection, PCR reaction was performed using Qiagen Taq PCR Kit (Qiagen) in a final reaction volume of 15 μl. The reaction mixture contained 1×Qiagen PCR Buffer (2.5 units Taq DNA pol, PCR Buffer with 1.5 mM $MgCl_2$, 200 μM dNTP mix), 0.66 μM *GAPDH* primers and DEPC-treated water up to 14 μl. Complementary DNA (1 μl) was added to the reaction volume. Cycling conditions were the same as for multiplex PCR.

PCR products were analyzed by electrophoresis on a 2% agarose gel (ethidium bromide-stained) and then were captured under UV

Table III. *Sequences of the assay primers.*

| Gene | Product size | Sequence |
|---|---|---|
| *CK19* | 159 bp | F: CGACTACAGCCACTACTACACGA |
| | | R: CTCATCCGCAGAGCCTGT |
| *CK20* | 371 bp | F: CAGACACACGGTGAACTATGG |
| | | R: GATCAGCTTCCACTGTTAGACG |
| *EGFR* | 480 bp | F: TCTCAGCCACATGTCGATGG |
| | | R: TCGCACTTCTTACACTTGCG |
| *GAPDH* | 471 bp | F: CATCACTGCCACCCAGAAGA |
| | | R: TCCACCACCCTGTTGCTGTA |

light in a KODAK EDAS 290 Imaging System (CareStream Health, Rochester, NY, USA).

The sensitivity of the multiplex PCR assay was estimated at 10 cancer cells per 3 ml of peripheral blood, after spiking experiments using the HT29 cell line (Figure 1). We normalized PCR conditions and primer concentrations in order to have the same sensitivity for all markers, and no signal in the sample of the control group. In our spiking experiments, serial dilutions of HT29 cells ($10^5$, $10^4$, $10^3$, $10^2$, 10, 1, 0 cancer cells) were added to tubes containing 3 ml of peripheral blood from a single healthy sample. Erythrocyte lysis, RNA extraction, cDNA synthesis and multiplex PCR reaction were performed as described above and the expression profile of each dilution, determined the sensitivity of the test.

*Statistics*. Statistical analysis was performed using the Statistical Package for the Social Sciences Predictive Analytics Software (SPSS PASW statistics, version 20.0; SPSS Inc., Chicago, IL, USA). A comparison between subgroups based on demographic, clinical and pathological characteristics for the expression of the *CK19, CK20, EGFR* and their combinations, was performed with statistical tests $\chi^2$ and Fisher's exact test. For comparisons of more than two groups the Kruskal–Wallis test was performed. The results were considered statistically significant if $p<0.05$.

## Results

*Expression of CK19, CK20 and EGFR in healthy controls, in patients with CRC and in patients with GC*. In the study 38 healthy volunteers, 81 patients with CRC and 16 patients with GC were enrolled. The expression patterns of *CK19*, *CK20* and *EGFR* in the peripheral blood of healthy controls and patients are presented in Table IV. Examples of the gel electrophoresis are presented in Figure 2.

*Comparison between healthy controls and patients with CRC*. The initial step in the statistical analysis was the comparison between patients with CRC and healthy controls regarding the expression of the *CK19*, *CK20*, and *EGFR* and their combinations. The expression of *CK19* seemed to be linked to the presence of CRC [odds ratio (OR)=8.54, 95% confidence interval (CI)=3.538-20.612; $p=0.0000004$]. This result reveals that a patient with CRC has an 8.54-fold increased probability of expressing *CK19*. Similar results were obtained from studying the expression pattern of *CK20* (OR=14.118, 95% CI=5.484-36.345; $p=0.000000002$), and from the combination of *CK19* with *CK20* (OR=10.667, 95% CI=3.976-28.614; $p=0.0000002$). Similarly, a patient with CRC has 14.118-fold increased likelihood of *CK20* expression and 10.667-fold of expression of the combination of *CK19* with *CK20*. The expression of *EGFR*, however, did not appear to be associated with the presence of CRC ($p=0.503$).

Table IV. *Expression patterns of cytokeratin 19 (CK19), cytokeratin 20 (CK20) and epidermal growth factor receptor (EGFR) mRNAs in the peripheral blood of healthy controls and patients with colorectal (CRC) or gastric cancer (GC).*

| Marker | Number (%) | | |
|---|---|---|---|
| | Healthy controls (n=38) | CRC (n=81) | GC (n=16) |
| CK19 | 10 (26%) | 61 (75%) | 15 (94%) |
| CK20 | 8 (21%) | 64 (79%) | 15 (94%) |
| EGFR | 4 (10%) | 17 (21%) | 6 (38%) |
| CK19 + CK20 | 2 (5%) | 54 (67%) | 14 (88%) |
| CK19 + EGFR | 2 (5%) | 14 (17%) | 6 (38%) |
| CK20 + EGFR | 0 (0%) | 17 (21%) | 6 (38%) |
| CK19 + CK20 + EGFR | 4 (10%) | 14 (17%) | 6 (38%) |
| None | 26 (68%) | 10 (12%) | 0 (0%) |

*Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of CK19 and CK20 for the diagnosis of CRC*. We calculated the sensitivity, specificity, PPV, and NPV of the expression of *CK19*, *CK20* and their combination for the diagnosis of CRC. The overall data are presented in Table V. The PPV of the expression of *CK19* for diagnosis of CRC was 85.9%, whereas that for *CK20* was 88.8%. Moreover, the specificity of the combinatorial expression of *CK19* with *CK20* was 94.7% and the PPV 96.4%.

*Associations between the tested markers and various clinicopathological parameters of patients with CRC*. We attempted to correlate the expression of *CK19, CK20, EGFR*, and their combinations with various clinicopathological parameters of patients with CRC. *p*-Values are presented in Table VI.

The statistical analysis of the results revealed no correlation between the expression of the studied markers and their combinations, and patients' gender, age (based on their median age of 70 years), segment of the large intestine where the tumor was located (right, left, rectum), maximum tumor diameter (classification according the median value of

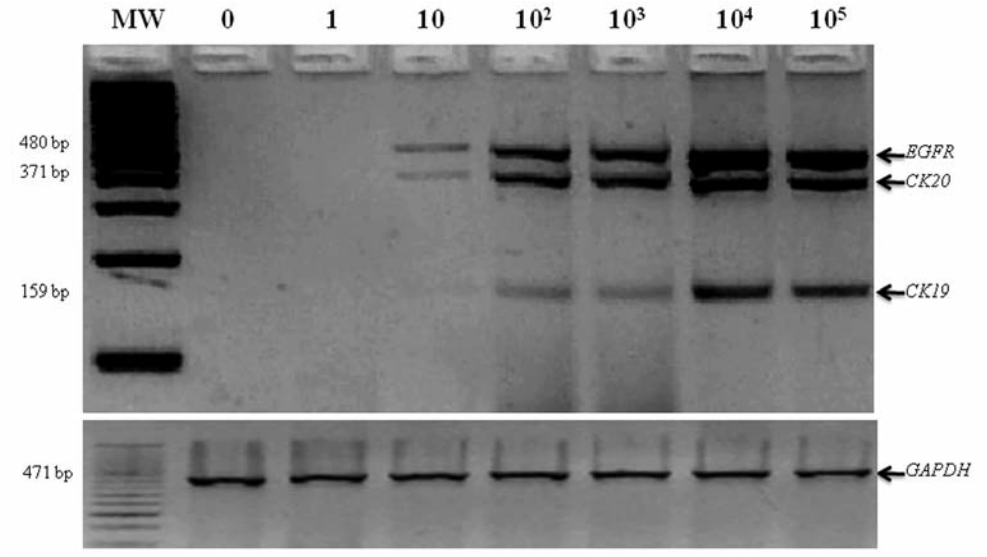


Figure 1. *Spiking experiments at densities of 0, 1, 10, 102, 103, 104 and 105 HT29 cells in 3 ml of peripheral blood of normal control volunteers. Epidermal growth factor receptor (EGFR), cytokeratin 20 (CK20) and cytokeratin 19 (CK19) produce a product size of 480, 371 and 159 bp, respectively. The sensitivity of our experimental method was set at 10 cancer cells per 3 ml of peripheral blood.*

Table V. *Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of cytokeratin 19 (CK19), cytokeratin 20 (CK20), epidermal growth factor receptor (EGFR), and their combinations for the diagnosis of colorectal cancer (CRC), the detection of distant metastasis in CRC patients and the diagnosis of gastric cancer (GC).*

| Marker | Sensitivity | Specificity | PPV | NPV |
|---|---|---|---|---|
| Diagnosis of CRC | | | | |
| *CK19* | 75.3% | 73.6% | 85.9% | 58.3% |
| *CK20* | 79% | 78.9% | 88.8% | 63.8% |
| *C19+CK20* | 66.6% | 94.7% | 96.4% | 57.1% |
| Detection of distant metastasis in CRC | | | | |
| EGFR (+CK20) | 63.5% | 83.6% | 29.4% | 95.3% |
| EGFR+CK19(+CK20) | 50% | 86.3% | 28.5% | 94% |
| Diagnosis of GC | | | | |
| *CK19* | 93.7% | 78.9% | 60% | 96.6% |
| *CK20* | 93.7% | 94.7% | 60% | 96.8% |
| *C19+CK20* | 87.5% | 94.7% | 87.5% | 94.7% |
| *EGFR+CK20* | 37.5% | 100% | 100% | 79.1% |
| *C19+CK20+EGFR* | 37.5% | 89.4% | 60% | 77.2% |

their maximum diameter, *i.e.* 4 cm), regional lymph node infiltration (N), grade (low or high), presence or absence of lymphovascular invasion and presence or absence of mucinous adenocarcinoma.

The stage of the disease was correlated with the expression of *EGFR*-alone ($p$=0.04) and in combination with the other markers (*CK19+EGFR*: $p$=0.039, *CK20+EGFR*: $p$=0.04, *CK19+CK20+EGFR*: $p$=0.039). The expression of CK19 and CK20 did not differ among the TNM stages of the disease. The comparison between metastatic and non-metastatic tumors confirmed the aforementioned correlation. The expression of *EGFR* and its combinations was significantly correlated with the presence of distant metastases (*EGFR*: OR=8.472 95% CI=1.781-40.295, $p$=0.009; *CK19+EGFR*: OR=6.3, 95% CI=1.353-29.332, $p$=0.027; *CK20+EGFR*: OR=8.472, 95% CI=1.781-40.295, $p$=0.009; *CK19+CK20+EGFR*: OR=6.3, 95% CI=1.353-29.332, $p$=0.027). Stage 4 tumors were 8.472-fold more likely to express *EGFR*, or *EGFR* and *CK20*, and 6.3-fold more likely to express *CK19* and *EGFR*, or *CK19* with *CK20* and *EGFR*.

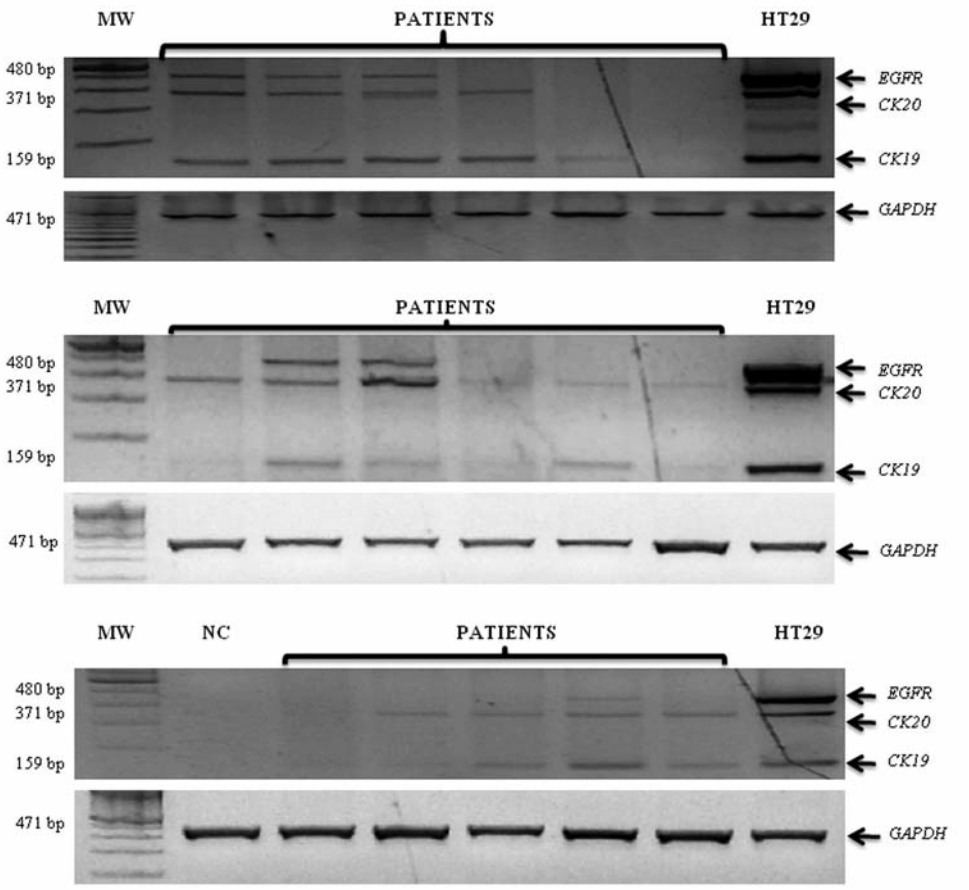


Figure 2. *Examples of positive polymerase chain reaction-based detections of cytokeratin 19 (CK19),cytokeratin 20(CK20) and epidermal growth factor receptor (EGFR) mRNAs in peripheral blood of patients with colorectal and gastric cancer. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. NC: Negative control.*

The expression of *CK19* and *CK20* did not differ between metastatic and non-metastatic CRC. No statistical correlation was revealed when we compared tumors with lymph node infiltration or distant metastasis (stages 3, 4) with the remaining tumors (stages 0, 1, 2) (*CK19*: $p$=0.588; *CK20*: $p$=0.240; *EGFR*: $p$=0.412; *CK19+CK20*: $p$=0.442; *CK19+EGFR*: $p$=0.761; *CK20+EGFR*: $p$=0.412; *CK19+CK20+EGFR*: $p$=0.761). Additionally, no significant difference was detected when we compared tumors that invaded up to the *muscularis propria* and had no lymph node metastasis (stages 0,1) with tumors that invaded beyond the *muscularis propria* or had metastasized to lymph nodes or distant sites (stages 2, 3, 4) (*CK19*: $p$=0.279; *CK20*: $p$=0.264; *EGFR*: $p$=0.815; *CK19+CK20*: $p$=0.087; *CK19+EGFR*: $p$=0.362; *CK20+EGFR*: $p$=0.815; *CK19+CK20+EGFR*: $p$=0.362).

The depth of tumor invasion did not seem to affect the expression of *CK19, CK20* and *EGFR*. No differences were revealed when we attempted to compare tumors that invaded up to the sub-mucosa with those that invaded beyond it (T0, T1 *vs*. T2, T3, T4: *CK19*: $p$=1; *CK20*: $p$=0.675; *EGFR*: $p$=0.66; *CK19+CK20*: $p$=0.485; *CK19+EGFR*: $p$=0.607; *CK20+EGFR*: $p$=0.66; *CK19+CK20+EGFR*: $p$=0.607). Additionally, no significant differences were observed between tumors that invaded up to the *muscularis propria* and those that invaded beyond it (T0,T1,T2 *vs*. T3,T4: *CK19*: $p$=0.091; *CK20*: $p$=0.271; EGFR: $p$=0.878; *CK19+EGFR*: $p$=0.344; *CK20+EGFR*: $p$=0.878; *CK19+CK20+EGFR*: $p$=0.344); with the exception of the marginally-significant increased expression of the combination of *CK19* and *CK20* in tumors invading up to the *muscularis propria* than those that invaded beyond it (*CK19+CK20*: OR=0.364, 95% CI=0.131-1.01; $p$=0.049). The expression of the markers did not differ when comparing tumors infiltrating the surface of serosa (T4a) or adjacent structures (T4b) with the remaining tumors (T0, T1, T2, T3 *vs*. T4: *CK19*: $p$=1; *CK20*: $p$=0.33; *EGFR*: $p$=0.293; *CK19+CK20*: $p$=0.658; *CK19+EGFR*: $p$=0.198; *CK20+EGFR*: $p$=0.293; *CK19+CK20+EGFR*: $p$=0.198).

Table VI. *p-Values for correlation of the expression of cytokeratin 19 (CK19), cytokeratin 20 (CK20), epidermal growth factor receptor (EGFR), and their combinations with various clinicopathological parameters of colorectal cancer patients.*

| Parameter | Marker | | | | | | |
|---|---|---|---|---|---|---|---|
| | *CK19* | *CK20* | *EGFR* | *CK19+ CK20* | *CK19+ EGFR* | *CK20+ EGFR* | *CK19+ CK20+ EGFR* |
| Gender | 0.938 | 0.967 | 0.249 | 1 | 0.170 | 0.249 | 0.170 |
| Age (MV:70 years) | 0.177 | 0.268 | 0.575 | 0.529 | 0.799 | 0.575 | 0.799 |
| Segment of the large intestine | 0.942 | 0.887 | 0.834 | 0.8 | 0.871 | 0.834 | 0.871 |
| Max tumor size (MV: 4 cm) | 0.467 | 0.535 | 0.519 | 0.573 | 0.306 | 0.519 | 0.306 |
| TNM stage | 0.673 | 0.724 | 0.04 | 0.4 | 0.039 | 0.04 | 0.039 |
| T (Tumor) | 0.128 | 0.358 | 0.711 | 0.066 | 0.188 | 0.711 | 0.188 |
| N (node) | 0.333 | 0.228 | 0.904 | 0.374 | 0.139 | 0.904 | 0.139 |
| M (Metastasis) | 0.673 | 0.724 | 0.009 | 0.4 | 0.027 | 0.009 | 0.027 |
| Grade | 0.116 | 0.764 | 0.527 | 0.13 | 1 | 0.527 | 1 |
| Lymphovascular invasion | 0.512 | 0.728 | 0.467 | 0.597 | 0.689 | 0.467 | 0.689 |
| Mucinus adenocarcinoma | 0.410 | 1 | 0.643 | 1 | 1 | 0.643 | 1 |

MV: Mean value.

*Sensitivity, specificity, PPV, NPV of EGFR and its combinations for the detection of distant metastasis in patients with CRC*. We calculated the sensitivity, specificity, PPV, NPV of *EGFR* and its combinations for the detection of distant metastasis in patients CRC (Table V). Patients with positive expression of *EGFR* were also positive for the expression of *CK20*. Moreover, patients who were positive for the expression of *CK19* and *EGFR* were also positive for CK20. The specificity of *EGFR* alone or in combination with CK20 was 83.6%, and NPV was 95.3%, whereas the specificity of the combination of *CK19* with *EGFR* alone, and in combination with CK20 was 86.3%, and NPV was 94%.

*Comparison between healthy controls and patients with GC*. The comparison of *CK19* expression between healthy individuals and patients with gastric adenocarcinoma revealed the positive correlation of this marker with the presence of the disease (OR=42, 95% CI=4.896-360.262; $p$=0.000006). Similar results were obtained for the expression of *CK20* (OR=56.25, 95% CI=6.427-492.3; $p$=0.0000008) and the combination of *CK19* with *CK20* (OR=37.333, 95% CI=6.691-208.308; $p$=0.0000006). Moreover, the expression of the combination of *CK20* and *EGFR* was also associated with the presence of GC, but with a marginal significance (OR=5.1, 95% CI=1.198-21.706; $p$=0.049). The same results were obtained from the study of the expression of the combination of all three markers, due to the fact that patients positive for *CK20* and *EGFR* were also positive for *CK19*. The expression of *EGFR* alone ($p$=0.148) or in combination with *CK19* ($p$=0.148) did not differ between patients and controls. Interpretation of statistical analysis indicates that patients with gastric adenocarcinoma have a greater likelihood of expressing *CK19* by 42-fold, *CK20* by 56.25-fold, the combination of *CK19* and *CK20* by 37.33-fold, *EGFR* by 5.1-fold, and of *CK20* and *EGFR* by 5.1-fold in comparison with healthy volunteers.

Table VII. *p-Values for correlation of the expression of cytokeratin 19 (CK19), cytokeratin 20 (CK20), epidermal growth factor receptor (EGFR), and their combinations with various clinicopathological parameters of gastric cancer patients.*

| Parameter | Marker | | | |
|---|---|---|---|---|
| | *CK19* | *CK20* | *EGFR* | *CK19+ CK20* |
| Gender | 1 | 1 | 0.608 | 0.467 |
| Age (MV: 67 years) | 1 | 0.438 | 1 | 1 |
| Segment of the stomach | n/a | 0.545 | 0.061 | 0.545 |
| Max diameter (MV: 4.3 cm) | n/a | 0.455 | 0.545 | 0.455 |
| TNM stage | 1 | 1 | 0.88 | 0.476 |
| T (Tumor) | n/a | 0.583 | 0.47 | 0.583 |
| N (node) | n/a | 0.333 | 1 | 0.333 |
| M (Metastasis) | 0.313 | 0.313 | 0.588 | 0.083 |
| Grade | n/a | 1 | 0.58 | 1 |
| Lauren's classification | n/a | 1 | 1 | 1 |
| Lymphovascular invasion | n/a | 1 | 0.242 | 1 |

MV: Mean value; n/a: not available.

*Sensitivity, specificity, PPV, NPV values of CK19, CK20 and EGFR for the diagnosis of GC*. We calculated the sensitivity, specificity, PPV, NPV of *CK19, CK20, EGFR* and its combinations for diagnosis of GC (Table V). The sensitivity of the expression of *CK19* for diagnosis of the disease was 93.7% and the NPV was 96.6%. The sensitivity of the expression of

CK20 was 93.7%, the specificity was 94.7% and the NPV was 96.8%. Moreover, the sensitivity of the combinatory expression of *CK19* and *CK20* was 87.5%, the specificity was 94.7%, the PPV was 87.5% and the NPV was 94.7%.

*Associations between the tested markers and various clinicopathological parameters of patients with GC*. The statistical analysis of the expression of *CK19*, *CK20* and *EGFR* examined possible associations with various clinicopathological parameters of patients with GC (Table VII). The study revealed no positive correlations. More specifically, no significant correlations were found between the expression of the markers and gender, age (based on the median value of 67 years), the segment of the stomach where the tumor was located, the maximum diameter of the tumor (based on the median value of 4.3 cm), TNM stage, T, N, M, its histological grade, the type of gastric adenocarcinoma according to Lauren's classification (intestinal or diffuse type) and the presence or not of lymphovascular invasion.

## Discussion

Early diagnosis of patients suffering from CRC or GC is essential for the success of therapeutic measures (5, 6). In addition, the detection of micrometastasis or residual disease is also of crucial importance for patients' survival. PCR-based CTC detection methods offer an additional, non-invasive and novel weapon in our diagnostic arsenal against cancer (16). The application of multiple molecular markers increases the specificity and sensitivity of the method, which however may be rather impractical (17, 18). A multiplex-PCR assay allows the rapid and cost-effective detection of CTCs.

The comparison of the expression of *CK19*, *CK20* and *EGFR* between healthy controls and patients with adenocarcinoma of the colon revealed a statistically significant correlation of *CK19*, *CK20* and their combination with the presence of disease. *EGFR* did not correlate with the presence of the disease. Similar results have also been reported from other studies, where the expression of *CK19* or *CK20* was elevated in patients with CRC in comparison to healthy individuals (14, 19-21). *EGFR* has also been found to be elevated in patients with CRC compared to healthy controls (11, 12). Specifically, a patient with colon adenocarcinoma has an 8.54-fold increased likelihood of expressing *CK19* when compared to a healthy individual, with a increased likelihood of 14.118-fold for *CK20* and 10.667-fold for the combination of *CK19* and *CK20*. The assessment of sensitivity, specificity, PPV and NPV of the expression of the aforementioned markers to detect CRC led to the following conclusions. Most people with positive expression of *CK19* or *CK20* will probably have the disease, due to the high PPV of these markers. The absence of the simultaneous expression of *CK19* and *CK20*, due to its high specificity, may be used for excluding the presence of the disease. Therefore, the vast majority of those who do not have CRC will also be negative for the combination of *CK19* and *CK20*. Moreover, the high PPV of the combination of *CK19* and *CK20* means that most people with positive results for the combination will have the disease. Nevertheless, the method is not capable of diagnosing colon adenocarcinoma, due to the low sensitivities of the markers.

The statistical analysis of the expression of *CK19, CK20* and *EGFR* and their correlations with various clinical, pathological and demographic characteristics of patients with CRC revealed the following results. The expression of *EGFR* mRNA in the peripheral blood of patients with adenocarcinoma of the colon, whether alone or in combination with the other markers (*CK19*, *CK20*), was statistically significantly correlated with the stage of disease. The remaining two markers did not appear to correlate with disease stage, although other investigators report the correlation of *CK19* and *CK20* with tumor stage (18, 19, 22, 23). Evidence from other studies supports the notion that *EGFR* is expressed in advanced cancer stages and is correlated with metastatic disease (12, 24, 25). The correlation was confirmed when we compared metastatic with non-metastatic tumors. Metastatic tumors (stage 4) were more likely to express *EGFR* (8.472-fold), CK19 and EGFR (6.3-fold), *CK20* and *EGFR* (8.472 fold) and the combination of all three markers simultaneously (6.3-fold). The study did not reveal any additional correlation when comparing tumors with lymph node or distant metastases (stages 3, 4) with tumors without spread beyond the primary site (stages 0, 1, 2). Additionally, there appeared to be no difference in the expression of markers when we compared tumors that invaded up to the submucosa without the presence of metastasis (stages 0, 1) with tumors with infiltration beyond the submucosa or with metastasis (stages 2, 3, 4). Statistical analysis revealed no correlation between T stage, and the expression of the studied markers and their combinations. Additionally, infiltration beyond the submucosal layer, *serosa intima* or adjacent organs does not affect the expression of markers. Surprisingly, while infiltration of the *muscularis propria* did not appear to show a statistically significant correlation with the expression of *CK19*, *CK20* and *EGFR*, tumors that invade beyond the *muscularis propria* (T3, T4) have borderline reduced likelihood of expressing the combination of *CK19* and *CK20*. Data from this study show that the expression of *CK19*, *CK20* and *EGFR* is not influenced by the gender or the age of the patients, by the location of tumors in the colon, the maximum diameter of the tumor, the presence of lymph node metastases, degree of tumor differentiation, lymphovascular infiltration, or mucinous adenocarcinoma. Our results oppose those of other research projects which have connected the expression of these markers with various clinicopathological

parameters (18-20). Statistical analysis was completed by determining the sensitivity, specificity, PPV and NPV expression of *EGFR* and its combinations in detecting distant metastases. According to these values, the expression of *EGFR* cannot be used to detect metastatic disease because of its low sensitivity and PPV. However, a negative result for *EGFR* can be used to exclude the presence of distant metastases, due to its high specificity and NPV. According to the above, the great majority of patients who do not have metastatic disease will not express *EGFR*, and the majority of patients negative for *EGFR* expression will not have distant metastases. The application of additional markers did not add any further diagnostic information.

Regarding gastric adenocarcinoma, data from other studies revealed the elevated expression of CTC markers in patients suffering from GC in comparison with healthy volunteers and its possible use for detecting GC (26-28).

In accordance to these results, the comparison of the expression of CK19, CK20, EGFR and their combinations between healthy controls and patients with gastric adenocarcinoma revealed the statistically significant correlation between the expression of *CK19, CK20, CK19* with *CK20, CK20* with *EGFR* and the combination of all markers and the presence of gastric adenocarcinoma.

The high sensitivity and NPV for *CK19, CK20* and *CK19* with *CK20*, the high specificity of *CK20* and *CK19* with CK20, as well as the high PPV of the combination of *CK19* with *CK20* suggest that these markers may be used for diagnosing or excluding the presence of GC. Additionally, the values of the above parameters for the use of the combination of *CK20* with *EGFR* or the combination of all three markers for the diagnosis of GC indicate that these combinations may also be used to exclude the presence of disease, due to the high specificity. Further statistical analysis in the group of patients with GC did not reveal any statistically significant correlation between the expression of the studied markers and various clinical, pathological and demographic parameters. The small number of samples may have influenced the outcome of this study. Similar results are reported from Matsumura *et al*., with the exception of vascular invasion (28). Our results did not agree with those of Huang *et al*., which correlated *CK19* and *CK20* expression in patients with GC with depth of invasion, lymph node metastasis and distant metastasis. The authors, however, did not report any relation between the expression of CK19 and CK20 and the age or the gender of the patients (26).

In conclusion, the aim of this study was the development of a rapid cost-effective multiplex-PCR assay for the diagnosis and the detection of systemic disease in patients with CRC or GC. Data from our study suggest that the combination of *CK19* and *CK20* could be used for excluding the presence of CRC, as well as diagnosing and excluding GC. Furthermore, the positive detection of *EGFR* could also be used for evaluating the presence of distant metastases in patients already known to have CRC. The era of translational medicine dictates the development of novel diagnostic and prognostic measures in order to aid conventional methods of monitoring patients with cancer and to identify patients in need of personalized and targeted therapeutic approaches. Further validation of the significance of multiplex PCR-based CTCs detection in disease progression-free survival and overall survival by larger-scale studies could prove the application of this assay "from bench to bedside".

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